Antifungal Effect of *Allium spp.* Root Exudates on Spore Germination of *Fusarium oxysporum* f. sp. *cubense*

ALDREAN PAUL E. ALOGON, JANIEL LLOYD J. HORLADOR, MATT JOSHUA S. MARTINETE, and HAROLD P. MEDIODIA
Philippine Science High School - Western Visayas Campus, Brgy. Bito-on, Jaro, Iloilo City 5000, Department of Science and Technology – Science Education Institute, Philippines

Abstract

Allium tuberosum root exudate has been proven to be effective against *Fusarium oxysporum* f. sp. *cubense* in field through crop-rotation, and subsequently in vitro, however is not economically advantageous in the Philippine market because of low consumer demand. The study done aimed to determine the antifungal effect of various *Allium* species on *Fusarium oxysporum* f. sp. *cubense* Tropical race 4. Root exudates (RE) from *Allium* species were tested against *Foc* tropical race 4. Exudates were collected by growing seedlings of each *Allium* species in 0.05 M $CaCl_2$ solution for 15 days and filtering the solution. Fungal mycelia were inoculated in potato dextrose broth (PDB), placed in a rotary shaker, and filtered and resuspended in PDB, where every 9 mL of the suspension was added 1 mL of treatment. Spore germination ratios in all experimental treatments were significantly lower than that in control distilled water, with *Foc* spore germination ratios of 54.6%, 54.9%, 58.8%, and 70.9% in chive RE, leek RE, onion RE, and Chinese leek RE, respectively. Thus, the tested *Allium* species show significant inhibitory effects on spore germination of *Foc* in the laboratory setting.

Keywords: *Fusarium oxysporum f. sp. cubense, Allium, spore inhibition, root exudates, germination ratio*

Introduction. The fungus *Fusarium oxysporum* f.sp. *cubense* (*Foc*) is the most significant disease agent of bananas (*Musa spp.*) worldwide [1] which induces plant wilting and death [2]. The infection resulted to a significant decrease to the Philippines' production of banana, which is the 2nd most exported agricultural product of the country [3]. *Foc* is particularly hard to eradicate because of its chlamydospores. Although no study has been done on the long-term survival rate of *Fusarium oxysporum* chlamydospores, it was shown that it could survive in dry conditions at 30℃ for 42 days [4].

Various methods of eradicating the fungus have been studied such as use of nanoparticles, volatile organic compounds (VOCs), and biocontrol agents [5,6,7], but all have only been subject to in vitro trials and have not yet been conclusively proven to work in field conditions. Another possible method is crop rotation with *Allium tuberosum* (Chinese leek) which was found to decrease the severity index of *Foc* Tropical race 4 (*Foc* TR4) by 91-96% in field trials [8]. The efficacy of the method is attributed to the sulfur-rich volatile compounds exuded by the roots to the soil. The in vitro design of the method [9,10] used extracted exudates to determine antagonistic effects on spore germination and mycelial growth of *Foc* TR4, which were found to be significantly antifungal.

Though it is known that Chinese leek root exudates have antifungal activity on *Foc* TR4 [8,9,10], the antifungal activities of other *Allium* species root exudates against the fungus have not yet been studied. *Allium cepa* (onion), *Allium ampeloprasum* (leek), and *Allium schoenoprasum* (chive) are plants of the same genus which are not only more economically significant in terms of volume and value of production in the Philippines [11] but also were shown to be effective in suppressing microbial growth in various bacteria and fungi [12,13,14,15], albeit in crude extract or oil forms.

This study aimed to determine the antifungal effect of various *Allium* species on *Fusarium oxysporum* f. sp. *cubense* Tropical race 4. It specifically aims to:

(i) Compute spore germination ratio (%) of *Foc* after application of *Allium cepa* root exudate, *Allium ampeloprasum* root exudate, *Allium schoenoprasum* root exudate, *Allium tuberosum* root exudate, distilled water, 0.05 M $CaCl_2$ and Mancozeb.

(ii) Compare spore germination (%) after application of *Allium cepa* root exudate, *Allium ampeloprasum* root exudate, *Allium schoenoprasum* root exudate, and *Allium tuberosum* root exudate, each with distilled water and Mancozeb.

The results approximate field conditions in the sense that the organisms' actual exudation of volatile compounds is the active biological process behind crop-rotation and intercropping. It is hypothesized that there will be a significant decrease in spore germination of Foc TR4 after application of the root exudates. The study is supporting evidence for possible real-life application of crop rotation by farmers with other *Allium* species.

Methods. Root exudates were collected by growing the seeds of *Allium spp* in water and 0.05 M $CaCl_2$ based on the study of Spiegel (2002). Spore germination ratio was measured upon application of treatment using a hemocytometer. The following are the specific details for the methodology.

Sample collection. *Foc* TR4 culture media was acquired from Stanfilco, Division of Dole Philippines

Inc. *A. tuberosum* seeds were obtained from Known-You Seeds Co Ltd., while *A. cepa* Red Colorado seeds from Condor™. Necessary reagents and chemicals for the study were obtained from the Philippine Science High School - Western Visayas Campus and Southern Mindanao Campus, while unavailable ones were purchased from local chemical and laboratory equipment distributors. The fungicide used was Mancozeb (Dithane® M-45 Neotec, Dow AgroSciences™).

Root Exudate Collection. Seeds were sterilized in 0.3% NaOCl solution for 5 min, washed thoroughly with sterilized distilled water three times, and transferred to 9-cm-diameter petri plates containing sterilized distilled water for germination. After 8 days of germination in the dark at ambient temperature, plantlets were transferred to 9-cm-diameter petri plates with 5 ml 0.05 M $CaCl_2$ solution for further growth in the dark at ambient temperature, with each petri plate having at most 100 plantlets. After 15 days, the root exudate solution was filtered using filter paper and stored at 4°C until use. Concentration of the extract for each species was derived as the total mass of combined seeds or seedlings per total volume of extracted root exudates [16].

Foc spore inoculation. Fungal mycelia were collected from a 23-day old *Foc* culture and inoculated into 50 ml potato dextrose broth (PDB) in a 500 ml Erlenmeyer flask, then were placed on a rotary shaker at 200 rpm when equipment was available or otherwise shaken manually every 1.5 hrs. After 4 days, spore suspension was filtered using a sterile gauze and resuspended in PDB. The conidial suspensions were adjusted to approximately 2 x 10^6 conidia/mL. 1 mL of exudate sample was mixed with 9 mL conidial suspension. After mixing, the mixture was homogenized with a vortex mixer. For the control, distilled water was used. The treated conidia were incubated at 28°C for 16 hours. [10]

Measurement of spore germination ratio. One drop of the suspension from each treatment was observed under a microscope using a hemocytometer. In each sample, the number of germinated conidia was counted in at least 300 observed spores. Three trials were done for each treatment. The germination ratio was calculated as a percentage, using the following equation [10]:

$$\text{Germination ratio (\%)} = \frac{\textit{Germinated conidia}}{\textit{Total conidia}} \times 100\%$$

Data Analysis. The sets of data were analyzed using the online program "Statistical tools for high-throughput data analysis" (STHDA, http://www.sthda.com/english/rsthda/unpaired-t-test.ph), and significant differences were determined using Student's t-test (P<0.05).

Safety Procedure. To sterilize *Foc* spore suspension, the Erlenmeyer flask was filled with 50 mL 10% NaOCl (Zonrox™ Bleach Original, Green Cross Inc.) along with 50 mL tap water, shaken slightly, and left stood for 10 mins. All contents were poured in an autoclavable bag and the bag together with the contaminated glassware were autoclaved. Glassware were then washed with soap. For the duration of the data gathering, nitrile gloves and N-95 facial masks were worn for precautionary measures.

Results and Discussion. Qualitative observation showed that the germinated spores were mostly, if not all, microconidia which were characterized by having oval to kidney shapes and one to two cells. The spore germination ratio in each treatment is shown in Figure 1. Spore germination ratio in experimental treatments were significantly lower than in distilled water. Compared to 81.9% spore germination in distilled water, only 54.6% of spores germinated in chive RE, followed by 54.9% in leek RE, 58.8% in onion RE, and lastly 70.9% in Chinese leek RE. Spore germination ratio in the positive control Mancozeb was significantly lower than all the experimental treatments. Although there can be seen an increasing order in the spore germination ratios in the experimental treatments, comparison cannot be made between them due to differences in their crude concentrations. The positive control Mancozeb induced the lowest germination ratio of 37.6% while the $CaCl_2$ the highest with 84.6%.

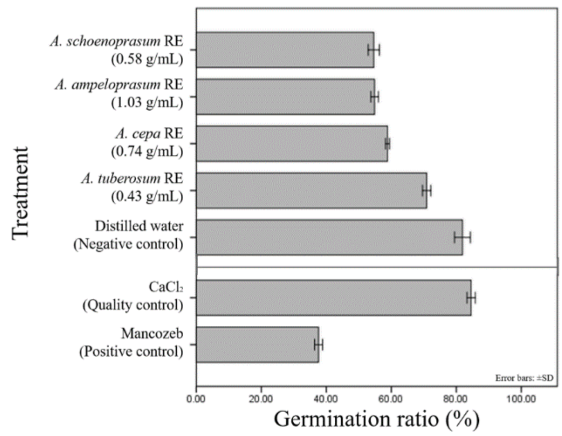

Figure 1. Treatments with their concentrations and corresponding spore germination ratio of *Foc* TR4 in the treatment. Concentration of active ingredient Mancozeb based on prescribed concentration by the supplier, i.e. 0.00536g/mL. Comparison was only done between an experimental treatment and the negative/positive control, rather than comparison among each other.

Foc TR4 spore germination ratios in the experimental treatments were all lower compared with that in distilled water. This may be evidence that these *Allium* species root exudates have their respective antifungal activity on *Foc* TR4 which was observed in the significant decrease of spore germination at their application. These findings are in line with in line with the findings of Gopi and Thangavelu [15], Rattanachaikonsupon and Phumchachorn [12], Sadeghi et al. [14], and Zhang et al. [9], which showed the antifungal effects of chive, leek, onion, and Chinese leek, however, leaf extracts or oil were used and were tested on different fungi. The results of this study support the literature in that the effects can be specified to *Foc* TR4 by root exudate, which was not established before. Although antifungal activity can be concluded for the experimental treatments, such activity is not comparable with the activity of an established effective fungicide, i.e. Mancozeb, and still falls short statistically. This may be caused by the low concentration of the exudates collected, or a weaker mechanism of action compared to the fungicide.

This study mainly adapted the methods of Zuo et al. [10] which studied the effects of aqueous root exudate extract on spore germination of *Foc* TR4, however with differences in the solvent used, the concentrations of root exudates, and the incubation period. The crude collected root exudates in $CaCl_2$ of the *Allium* species in this study had concentrations less than or equal to 1 g/mL, less than the 2 and 8 g/mL aqueous extract used by Zuo et al. [10]. The $CaCl_2$ control was not significantly different in terms of its spore germination inhibition percentage compared distilled water treatment in the results, thus the difference can be neglected. Comparing Chinese leek results in this study with that of Zuo et al. [10] shows a wide disparity between the two results, 70.9% and <10%, respectively. Both the deviations in concentrations and the incubation period (Zuo et al.: 2 & 8 g/mL, 1-6 h; study: ≤1 g/mL, 16 h, respectively) are plausible causes of the disparity, with the latter analytically contributing more. After the 16 h incubation period, only 81.9% of spores germinated in distilled water, compared to the 6 h incubation by Zuo et al. [13] with 28.2% of spores germinated. The relatively high germination ratio in the negative control treatment (37.6%) could be caused by either the longer incubation period, the inefficacy of the fungicide, or the difficulty of the total eradication of *Foc* spores itself. Nevertheless, all the treatments were significantly effective in inhibiting spore germination. In field, application of the root exudates on soil through the process of crop-rotation is predicted to significantly decrease the disease severity [8] which is defined as the number of banana plants infected over total number of plants. Possible mechanism of action of the *Allium* root exudates may be highly similar with that of the Chinese leek root exudates, which namely are the induction of accumulation of reactive oxygen species (ROS), a decrease transmembrane potential, and a decrease of expression in ergosterol synthesis genes [10].

Conclusion. The study has successfully done the general objective of determining the antifungal activity of onion, chive, and leek root exudates on *Foc*, and from the results, the effect is highly significant and therefore support the effectiveness of crop rotation in field of the tested *Allium* species with bananas in decreasing disease severity. It further supports the idea that *Allium* root exudates could be effective in controlling the Foc both in vivo and in vitro. The study provides evidence to support the practical application of crop-rotation on Foc infested banana fields with onion, chive, and leek which have higher economic demand in the country.

Recommendations. To supplement the existing study, it is recommended for future studies to study the correlation of the antifungal activity of root exudates with varying concentrations, to determine the optimal concentration at which statistically all fungi spores are inhibited, and with time, to be able to answer the question of how immediate the activity is, how long it will last, and at what time its peak inhibition will be. A comprehensive detailing of the composition of root exudates for the *Allium* genus is also suggested to verify if there is a common compound/s behind their efficacy.

Acknowledgement. We thank Stanfilco, Dole for their generosity in providing our culture, and Philippine Science High School – Southern Mindanao Campus for accommodating us during our stay in Davao.

References

[1] Ploetz RC. 2006. Fusarium wilt of banana is caused by several pathogens referred to as *Fusarium oxysporum* f. sp. *cubense*. The Amer Phytopathol Soc. 96(6):653-656.

[2] Yin XM, Xu BY, Zheng W, Wang Z, Wang BZ, Ma WH, Mao HT, Li JY, Sheng ZW, Wang DX, He YD, Han LN, Chen SW, Zhang ZB, Fu YG, Wang JB, Jin ZQ. 2011. Characterization of early events in banana roots infected with green fluorescent protein-tagged *Fusarium oxysporum* f. sp. *cubense*. Acta Horticul. 897.

[3] PSA (Philippine Statistics Authority). 2016a. Philippine agriculture in figures. [Internet]. [Cited 26 February 2018.] Available from http://countrystat.psa.gov.ph/?cont=3

[4] Bennet RS. 2012. Survival of *Fusarium oxysporum* f. sp. *vasinfectum* chlamydospores under solarization temperatures. Plant Dis. 96: 1564-1568.

[5] Smolinska U, Morra MJ, Knudsen GR, James RL. 2003. Isothiocyanates produced by *Brassicaceae* species as inhibitors of *Fusarium oxysporum*. Plant Dis. 87:407-412.

[6] Herbert JA & Marx D. 1990. Short-term control of Panama disease in South Africa. Phytophylactica. 22:339–340.

[7] Thangavelu R & Mustafa M. 2012. Current advances in the Fusarium wilt disease management in banana with emphasis on biological control. in C. J. Cumagun (ed.), Plant Pathology. InTech. 273–298.

[8] Huang YH, Wang RC, Li CH, Zuo CW, Wei YR, Zhang L, Yi GJ. 2012. Control of Fusarium wilt in banana with Chinese leek. Eur J Plant Pathol. 134(1), 87–95.

[9] Zhang H, Mallik A, Zeng RS. 2013. Control of Panama disease of banana by rotating and intercropping with Chinese chive (*Allium tuberosum* rottler): role of plant volatiles. J Chem Ecol.

[10] Zuo C, Li C, Li B, Wei Y, Hu C, Yang Q, Yang J, Sheng O, Kuang R, Deng G, Biswas MK, Yi G. 2015. The toxic mechanism and bioactive components of Chinese leek root exudates acting against *Fusarium oxysporum* f. sp. *cubense* tropical race 4. Eur J Plant Pathol 143:447-460.

[11] PSA (Philippine Statistics Authority). 2016b. Selected Statistics on Agriculture [Internet]. [Cited March 17, 2018]. Available from https://psa.gov.ph/sites/default/files/Selected%20Statistics%20on%20Agriculture%202016.pdf

[12] Rattanachaikumsopon P & Phumchakorn P. 2008. Diallyl sulfide content and antimicrobial activity against food-borne pathogenic bacteria of chives (*Allium schoenoprasum*). Biosci Biotechnol Biochem. 72(11):2987-2991.

[13] Cornago DF, Amor EC, Rivera WL. 2011. Antifungal activity of onion (*Allium cepa* L.) bulb extracts against *Fusarium oxysporum* and *Colletotrichum* sp. Philipp Agric Scientist. 94(1):78-82.

[14] Sadeghi M, Zolfaghari B, Senatore M, Lanzotti V. 2013. Antifungal cinnamic acid derivatives from Persian leek (*Allium ampeloprasum* Subsp. *Persicum*). Phytochemistry Letters. 5:350-363.

[15] Gopi M & Thangavelu R. 2014. Suppression of Fusarium wilt disease of banana by Zimmu (*Allium cepa* L. x *Allium sativum* L.) leaf extract. African J. Microbiol. Res., 8(31):2904-2915.

[16] Spiegel Y, Burrows PM, Bar-Eyal M. 2003. A chemo attractant in onion root exudates recognized by *Ditylenchus dipsaci* in laboratory bioassay. Amer Phytopath Soc. 93(1):127-132.

.